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STUDIES OF ALTERED RESPONSE TO INFECTION INDUCED BY
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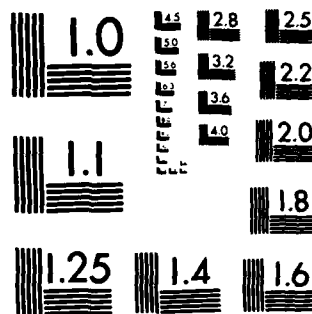
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STUDIES OF ALTERED RESPONSE TO INFECTION INDUCED BY THERMAL INJURY

ANNUAL PROGRESS REPORT

Carol L. Miller, Ph.D.

January, 1980

AD A140054

Supported by

U.S. Army Medical Research and Development Command

Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17 - 77 - C - 7012

University of California
San Francisco, California 94143

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A140054	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Studies of Altered Response to Infection Induced by Thermal Injury		5. TYPE OF REPORT & PERIOD COVERED Annual - 1/1/79-12/31/79
7. AUTHOR(s) Carol L. Miller, Ph.D.		6. PERFORMING ORG. REPORT NUMBER
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of California 1483 4th Avenue San Francisco, CA 94143		8. CONTRACT OR GRANT NUMBER(s) DAMD17-77-C-7012
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick, Frederick, MD 21701		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62772A.3S162772A874.AD.127
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE January 1980
		13. NUMBER OF PAGES 23
		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited.		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number)		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number)		



ARMY CONTRACT ANNUAL PROGRESS REPORT

Contract #DAMD 17-77-C-7012

Introduction

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The high incidence of fatal septicemia associated with severe thermal injury is believed to result from loss of immunocompetence. This laboratory has been able to identify those burn patients who are at greatest risk for developing fatal sepsis by detecting the loss of certain immune functions by cells of these patients. Direct burn induced immune dysfunction can result from aberrations in any of the three general types of leukocytes which cooperatively mediate the generation of immune function. These three leukocyte subpopulations are the antigen specific bone marrow-derived (B) cell, the antigen specific thymus-derived (T) cell, and a third extremely heterogenous population of leukocytes - the monocyte or macrophage (\emptyset).

This report describes the results of this year's experiments to reduce the post burn incidence of fatal sepsis by (1) rapidly identifying and segregating those individuals that are at greatest risk of sepsis; (2) delineating the nature of the burn induced immune defect; and (3) characterizing those mechanisms by which thermal injury causes immune aberrations. Understanding of these mechanisms may allow development of far forward prophylactic measures which could prevent thermal injury from inducing immune defects. Experimental data derived from our patient studies have allowed us to develop assays for detecting early immune anomalies and to delineate the cell type(s) involved in these aberrations. Our murine model has been primarily utilized to characterize the mechanisms by which thermal injury causes the development of immune defects.

One of the thrusts of this year's research was to develop a simple assay which would detect any early monocyte defects occurring after thermal insult. We have previously demonstrated that total loss of immunocompetence in burn patients was a result of the development of suppressor T lymphocytes (1,2). It has been demonstrated in murine systems and suggested for human systems that the preferential development of suppressor T lymphocytes can result from improper monocyte triggering of helper T cells (3-6). Additionally, the \emptyset has been shown to be the target cell of at least one type of human T suppressor cell (7). Consequently, the assessment of changes in monocyte function after thermal insult measures an extremely important parameter in the development of reduced resistance after burns. Reports utilizing murine systems suggested that monocyte production of plasminogen activator (PA), may be an accurate indicator of \emptyset function in both the nonspecific inflammatory and specific immune responses (8 - 10). Decreased monocyte PA activity would severely affect the post-burn balance between coagulation and fibrinolysis (coag-fibro) systems. Disruption of the coag-fibro systems would greatly affect the inflammatory and hence the immune functions of monocytes. As can be seen in Figure 1, any reduction in \emptyset function as a result of alterations in either the specific immune or the inflammatory systems will eventually affect most other host defense systems because of the central role of \emptyset functions in these systems. We have, therefore, assessed a variety of \emptyset functions after severe

burns and trauma concomitant to our measurement of the patient's immune function.

Methods

Human studies

Patients with greater than 20% full thickness burns are the primary donors of abnormal leukocytes. Leukocytes are obtained by venipuncture from consenting patients. Normal volunteers are donors of control human leukocytes. Appropriate safety precautions are always observed. Minors, prisoners, pregnant women and the mentally handicapped are excluded as donors. Mononuclear cells are isolated from the peripheral blood (PB) by Ficoll-Hypaque gradient centrifugation (11). Patient mononuclear populations can be further depleted of T cells, monocytes and/or B cells. The T lymphocytes are depleted by removing the cells binding to neuraminidase treated sheep red blood cells (SRBC) on a Ficoll-Hypaque gradient (12). Monocytes are removed by passing the mononuclear population over Sephadex G-10 columns (13). The B cells can be removed by nylon wool filtration of the cell population (14).

We monitor the ability of patient and normal mononuclear cell populations to repond to phytohemagglutinin (PHA) (15). This non-specific mitogen response requires the cooperative interaction of monocytes and T cells (16).

Monocytes are isolated from the Ficoll-Hypaque purified mononuclear cell populations by the Ackerman and Douglas rapid adherence technique (17). These isolated monocytes are then examined for the production of PA, their level of tissue procoagulant factor (TF) activity and their synthesis of lysozyme. In the PA assay, patients' and normal controls' isolated \emptyset are placed onto 125 I-fibrin plates and cultured 18 hours either in the presence of acid treated fetal bovine sera (AT-FBS) or soybean trypsin inhibitor (SBI), an inhibitor of plasmin. After all the PA is released in these cultures, the cells are washed and fresh AT-FBS media or SBI media is added for an additional 24 hour incubation period. The amount of fibrinolysis intitiated during this second incubation period is then measured. Monocyte numbers have been adjusted to produce approximately 25-35 fibrinolytic units for normal individuals (4×10^5 isolated \emptyset). Simultaneous to our assessment of burn patients monocyte's PA synthesis, we also assay their production of TF and lysozyme. TF production is measured using the Rickle's assay and lysozyme production is measured using the Schill and Schumacher Lysozyme Plate test (18). A diagram of our experimental design is presented in Figure 2.

We have detected burn induced aberrations in the immune regulatory functions of patients' cells using a modification of the classical mixed lymphocyte response (MLR). In our MLR system a highly responsive combination of cells from two normal individuals are cultured in a "one way" MLR (19). In this assay, one of the normal's cells are pretreated with mitomycin C (MC) to prevent their division (15). Consequently, this "one way" MLR assay measures the ability of one normal's cells (Responder= R) to proliferate in response to the foreign histocompatibility antigens on another normal's cells (Stimulator=S). We compare the effect of adding either burn patient cells or

MC treated responder cells, on the amount of proliferation in the MLR cultures.

Data Calculation

The data presented for patient and normal's PA production is always from the second incubation interval. All supernate CPM's of ^{125}I -fibrin are corrected for media and non-specific radioactivity release by subtraction of CPM's from no cell controls. The CPM's of ^{125}I -fibrin in the supernates from the lines containing cells in 100 ug SBI are subtracted from the CPM's of lines containing the cells in AT-FBS. This corrects for any ^{125}I -fibrin lysed by other than plasmin mediated mechanisms. This corrected AT-FBS CPM is then divided by the total ^{125}I -fibrin CPM's present to derive the percent specific plasmin mediated lysis. This value is computed for patient cells collected every four days post injury. The mean and standard deviation of PA production by \emptyset from 43 normal individuals tested repeatedly was 25 ± 6.3 units. All patients' PA data was compared to this normal value, the value for surgical controls of 24 ± 8.4 , and their own initial (day 1) values. A Student's T test was used to determine significant differences. The TF activity of sonicates from $10^5 \emptyset$ was calculated in thromplastin equivalent units by comparison of the shortened thromboplastin time to a control brain thromboplastin standard curve.

Murine experiments

In each experiment, BDF₁ or C57Bl/6 inbred mice of 16-18 weeks of age are obtained from the Diablo Mouse Colony at the University of California at Berkeley. Under light Metofane anesthesia, littermate mice are shaven and then divided into two groups. One group receives a 10-20% scald burn with 95°C water for 5 seconds (experimental mice) while the second group is not burned (sham-control mice). At specific times after injury, 2 - 4 mice from each group are sacrificed. These animals' spleens are removed, teased into single cell suspensions and cultured in vitro with sheep erythrocytes using a modification of the Mishell-Dutton culture technique (20). We monitor thermal injury effects on immunocompetence by measuring the formation of specific antibody forming cells (AFC). This system facilitates detection of cell immunoregulatory interactions.

Monocytes and or T leukocytes are depleted or isolated from the burned mice. Purified, syngeneic, normal or control leukocytes are added to these depleted thermally injured populations. In this manner, normal monocytes or T cells are supplied to the immunodepressed burned mouse's cells. These experiments examine whether supplying normal, functional \emptyset or T cells restore the ability of the thermally injured leukocyte population to generate normal numbers of specific AFC. \emptyset are depleted from leukocyte populations according to the method of Ly and Mishell (13). T cells are lytically removed from leukocyte populations by treatment of the splenocytes with anti-T cell antisera and complement (21). Leukocyte populations are depleted from splenocyte populations by passing the cell preparations over nylon wool columns (22). These nylon wool columns remove \emptyset as well as B cells.

The in vitro generation of AFC is assayed using the slide modification

of the Hemolytic Plaque Assay (23). Leukocyte recovery from cultures is determined by counting a sample of the harvested, cultured cells on a Coulter Counter (Model ZH). The number of AFC are calculated for each pool of duplicate background plaques and expressed as AFC/ 10^6 recovered spleen cells. Allogeneic conditioned media is produced as described (24). In order to augment ϕ function, 2-mercaptoethanol (2ME) is sometimes added to cultures at a final concentration of 5×10^{-5} M.

Murine splenocytes were simultaneously assessed for their ability to make an in vitro primary AFC response and the ability of the isolated monocytes to produce PA.

Results

This year we have extensively studied 7 patients in the 20-50 year age group who sustained greater than 30% 3^o burns. We also have examined 27 patients who had undergone splenectomy for trauma, 15 of whom had additionally sustained extensive soft tissue injury. We also assayed cells from 13 surgical controls and 43 normal individuals. We have previously demonstrated that burn patients experiencing PHA hyporesponsiveness concomitantly developed excessive T suppressor lymphocyte activity (1). We have also demonstrated that certain splenectomized and severely traumatized patients experience PHA hyporesponsiveness due to the development of inhibitory ϕ activity (25,2). When we examined the isolated monocytes from the severely burned patients, we found that some patients' monocytes lost their ability to produce PA at approximately 3 - 4 days post injury. These were the same patients that became mitogen hyporesponsive. The data on mononuclear cell mitogen responsiveness and on monocyte reactivity for a burn patient who succumbed to fatal septicemia are illustrated in Figure 3. Figure 4, in contrast, illustrates data from a burn patient who had an uncomplicated clinical course. As can be seen (Figure 3), the patient who eventually succumbed to fatal septicemia experienced a loss of ϕ function and a depression of his PHA response. Interestingly, the ϕ TF production in this patient is not maximally elevated until quite late in his clinical course. Since ϕ TF production is highly stimulated by endotoxin, this late rise in TF may reflect the patient's septic state, rather than an early aberrance in his ϕ function. However, the loss of PA production in this patient preceeds the onset of septic complication by 8 days and parallels or possibly slightly preceeds the development of PHA hyporesponsiveness. This pattern of PHA hyporesponsiveness and reduced ϕ PA function was seen in all those patients who later developed major septic complications (Figure 5). The patient PHA and PA responses are significantly ($p < 0.02$) different on day 1 and 5 as derived from comparison in the Wilcoxon test. Based on these data, we are exploring ϕ PA production as a probe for monocyte function in the immune response. We feel that early post burn changes in monocyte function may play a major role in the unbalancing of the immune response which eventually results in the development of suppressor T lymphocytes.

Another group of severely injured patients studied were those splenectomized for traumawith multiple soft tissue injury. In this patient group, monocyte TF production seems to play a more central role in the development of subsequent post injury pathological complications. In this patient group

there is a correlation between increased TF production, decreased PA activity, and development of thromboembolic complications (Fig. 6). As can be seen in Figure 7, some splenectomized trauma patients show a loss of \emptyset PA function and a development of PHA hyporesponsiveness concomitant to an enormous increase in \emptyset TF activity (significant at $p < .001$ vs surgical controls T test). The greatest reduction in patients' \emptyset PA (2-10 fibrinolytic units vs 25-30) occurred at 7 - 12 days post trauma. The maximal reduction in PHA occurred at 10 - 15 days post injury. Those patients with such reduced \emptyset PA, PHA hyporesponsiveness and augmented TF production always experienced thromboembolic complications. As can be seen in Tables 1 and 2, those patients who had increased \emptyset TF production also showed increased fibrin deposition as measured by detection of increased fractional catabolic rate (FCR). All of those trauma patients with greatly elevated \emptyset TF had clinically manifested hypercoagulable episodes (Table 2). Only those splenectomized trauma patients who experienced thromboembolic complications had monocytes with reduced PA production and increased TF production. Splenectomized trauma patients who had uneventful clinical courses had no significant changes in PA or in \emptyset TF when compared with amounts produced by monocytes from surgical controls (PA = 28 units, TF = 12 units). These data all seem to support our hypothesis that an early monocyte defect results in unbalancing both nonspecific inflammatory and specific immune host defense systems. In an additional set of experiments, we have attempted to show that the PA producing monocyte population also includes those monocytes which are necessary for initiation of the immune response. Such facilitory monocytes are the target of Concanavalin A induced T suppressor cells (7,26). These Con A induced T suppressors prevent initiation of immune responses by interfering with proper monocyte interaction with T helper cells and antigen. As illustrated in Table 3, Con A activated suppressor T cells will inhibit \emptyset production of PA as well as \emptyset facilitation of immune responses. This is the first direct evidence of T suppressor cells regulating an inflammatory response. These results open up new possibilities for investigating mechanisms by which burns can reduce host resistance. The monocyte appears to be one of the initial cell targets of burn induced immunoincompetence. Monocytes are also particularly vulnerable to changes in the levels of activated products of the complement system and coagulation cascade. Profound alterations in the plasma-proteins of the complement system and coagulation cascade occur after thermal insult. Post burn changes in these plasma protein effector systems could adversely effect monocyte functions. This aberrant monocyte activity could then affect both inflammatory and specific immune systems, unbalancing the specific immune system toward increased suppressor T cell activity. This increased suppressor T cell activity would further reduce monocyte functions thereby intensifying the pathological consequences of the burn induced alterations. We are attempting to dissect the temporal relationships between aberrant \emptyset function and immune dysfunction utilizing our more sophisticated murine system.

The availability of more sophisticated techniques and genetic information in the murine antibody forming cell system (AFC) has allowed us to more closely characterize the burn induced immune defect. As we have previously demonstrated, a 20% scald burn severely depresses development of a de novo antibody forming cell response in mice. This defect is not the result of a bursal equivalent cell defect. The B cells from burned mice can produce antibody normally in

cooperation with normal syngeneic T cells and ϕ . We have already demonstrated that there is an increase in T suppressor activity in the splenocyte populations from thermally injured mice (27). These burn induced T suppressor cells can prevent induction of a primary in vitro AFC response. We have designed our murine experiments to determine if aberrant macrophage activity in the murine splenocyte population precedes, coincides, or follows development of the AFC dysfunction. Results of these preliminary murine experiments are illustrated in Figure 8.

Loss of ϕ PA function does occur in the splenic population from thermally injured mice. The loss of macrophage PA function appears at 1 - 2 days post burn, while the development of murine suppressor T lymphocytes does not occur until 4 - 5 days after thermal insult. Additionally, only those animals who first experience defective ϕ function go on to develop extravagant suppressor T cell activity. We are currently attempting to demonstrate that a ϕ population whose PA function is abrogated is also dysfunctional in its antigen interactive function. These experiments will add greater credence to our hypothesis that monocyte dysfunctions are pivotal in the loss of immunocompetence after thermal injury.

Discussion

The data obtained in this contract year has provided an important illumination of one mechanism by which burns can cause immune anomalies. These data have led us to hypothesize that one means by which thermal injury decreases host resistance is to directly or indirectly cause aberrant monocyte function. Thermal injury initially causes abnormal activations of the complement system and elevation of steroid levels. These changes in the serum protein effector systems either alone or in combination with other events, adversely affect early monocyte inflammatory functions. This disruption in macrophage function causes further changes in the actions of the coagulation-fibrinolytic and complement systems. Those burn patients who are unbalanced too far toward aberrant ϕ function develop immune disarrangement due to the loss of ϕ facilitory functions and the excessive activation of suppressor T cells. These suppressor T cells further disrupt normal monocyte function intensifying the reduction of both inflammatory and specific immune host defense systems. The eventual result is a critically decreased resistance to infection. The first step to validating this hypothesis has been taken this year. We have shown that early post burn monocyte functions were abnormal in those critically burned patients who later succumbed to massive septicemia but not in burn patients who would have uncomplicated clinical courses. Monocyte PA function is known to parallel monocyte immune function. We have shown that loss of monocyte PA function in burn patients can be correlated with development of PHA hypo-responsiveness and appearance of extensive T suppressor activity. Additionally, we have demonstrated that this loss of monocyte PA function is not due to cell death since the monocytes' lysozyme and TF activities are not decreased. We have also produced data showing that once suppressor T lymphocytes are generated (ie, Con A T suppressors), these T suppressors can further reduce monocyte PA activity intensifying the burn induced inimical effects on monocyte function.

The murine system experiments have allowed examination of the relationship between macrophage function in induction of a specific immune response and \emptyset PA function. In these experiments we use the primary in vitro generation of antibody forming cells as a measure of the ability of \emptyset - T and B cells to cooperatively interact in response to foreign protein. The AFC response is totally abrogated in severely burned mice at approximately 5 - 7 days post injury (27,28). We have also previously demonstrated in this system that a 20% full thickness burn will result in the excessive development of suppressor T lymphocytes. This contract period we have demonstrated that a 20% third degree burn causes the loss of PA function by murine splenic macrophage. This loss of \emptyset PA function appears at 1-2 days post burn, while development of murine T suppressor lymphocytes does not occur until 4-5 days after thermal insult. Additionally, only those animals who first experience defective \emptyset function go on to develop excessive suppressor T lymphocyte activity. These data appear to support several parts of our hypothesis. Monocyte dysfunction does occur after thermal injury and precedes the development of immune aberrations. Once suppressive T lymphocytes have been generated they can further disrupt monocyte inflammatory activity. We are presently investigating the effect of post burn sera on monocyte systems, the role of elevated post burn steroid levels in depressing the host defense systems, and the relationship between the facilitory monocyte and the PA producing or prostaglandin producing monocyte.

Conclusions

↓ In summary, our laboratory has substantial data indicating that a monocyte defect does appear in severely burned patients who will eventually develop severe or fatal septicemia. This loss of patient monocyte function appears to correlate with a development of excessive T suppressor cells and mitogen hyporesponsiveness. Our preliminary data from a murine system seem to support that unbalancing of the immune network toward too much regulation (ie, increased T suppressor cells) is preceded by, and is probably a result of, loss of monocyte function. ↑

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Table 1

Correlation of Increased Fibrin Deposition and Increased Monocyte
of Tissue Procoagulant Factor (TF)

Moderate Trauma							
PT	Max increase TF(eq units)		% increase FCR(%/day)			Max increa	
	vs Norm	vs Surg Control	vs Norm	vs Surg Control		PT	vs Norm
S ₁	7	- 1	42	21		S ₂	41
K ₁	8	0	67	43		M ₁	61
B ₁	4	- 4	40	20		M ₂	41
B ₂	- 1	-10	13	- 3		B ₃	36
						F ₂	39
$\bar{X} \pm SD$	5 \pm 4	- 4 \pm 4	41 \pm 19	20 \pm 16		$\bar{X} \pm SD$	43 \pm 9

Table 1

Deposition and Increased Monocyte (ϕ) Production
Procoagulant Factor (TF)

PT	Severe Trauma			
	Max increase TF(eq units)		% increase FCR(%/day)	
	vs Norm	vs Surg Control	vs Norm	vs Surg Control
S ₂	41	33	136	102
M ₁	61	53	81	55
M ₂	41	33	70	46
B ₃	36	28	98	70
F ₂	39	31	107	78
$\bar{X} \pm SD$	43 \pm 9	35 \pm 9	98 \pm 23	70 \pm 19

2

Table 2

Production of TF by ϕ as Compared to Other Indices of Hypercoagulability

PT	TF Production of > 20 units over Surgical Control		Other Indicators of Hypercoagulability			
	<u>Amt increase</u> eq units	<u>Max increase</u> day	<u>Max FDP</u> titer	<u>day of app</u>	<u>Max monomers</u> amt	<u>day</u>
B ₁	38	13	128	24	3+	24
M ₂	33	13	16	14	2+	14
F ₁	31	7	32	6	1+	6
M ₁	53	9	64	12	N.D.	N.D.
S ₂	32	14	64	20 [*]	neg	neg [*]

* First done day 20.

er Indices of Hypercoagulability

Other Indicators of Hypercoagulability

of app	Max monomers		Clinical manifestations	
	amt	day	type	day
24	3+	24	Subphrenic abscess	25
			DVT	29
14	2+	14	Pulmonary infarct	16
6	1+	6	Leg Scan (+)	7
12	N.D.	N.D.	DVT	12
			Staph septicemia	20
20*	neg	neg*	Phlebitis	20

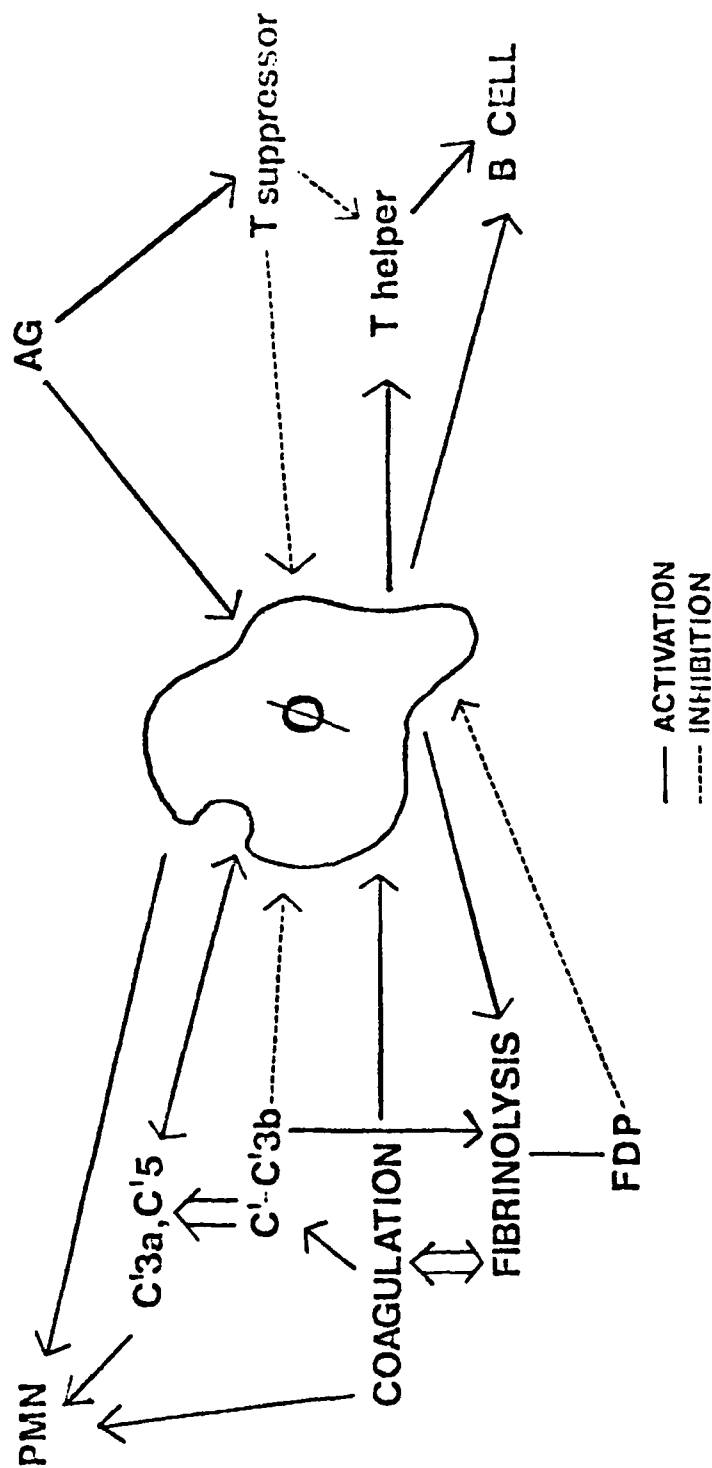
Table 3

Suppressor Cell Depression of Monocytes' (\emptyset) Production of Plasminogen Activator*

PA Produced by 4×10^5 Normal \emptyset after Exposure to 2×10^5 Autologous Cells					
Added Cells:	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5
Suppressor (Con A induced)	10.8	12.4	11.3	12.1	4.3
Cultured (Con A control)	22.3	38.6	22.8	36.2	15.7
Fresh	27.2	25.3	27.3	----	13.8

* PA production expressed as fibrinolytic units.

MONOCYTE CONNECTION OF INFLAMMATORY & IMMUNE DEFENSE SYSTEMS



Figure]

MEASUREMENT OF MONOCYTES' PRODUCTION OF PA-TF

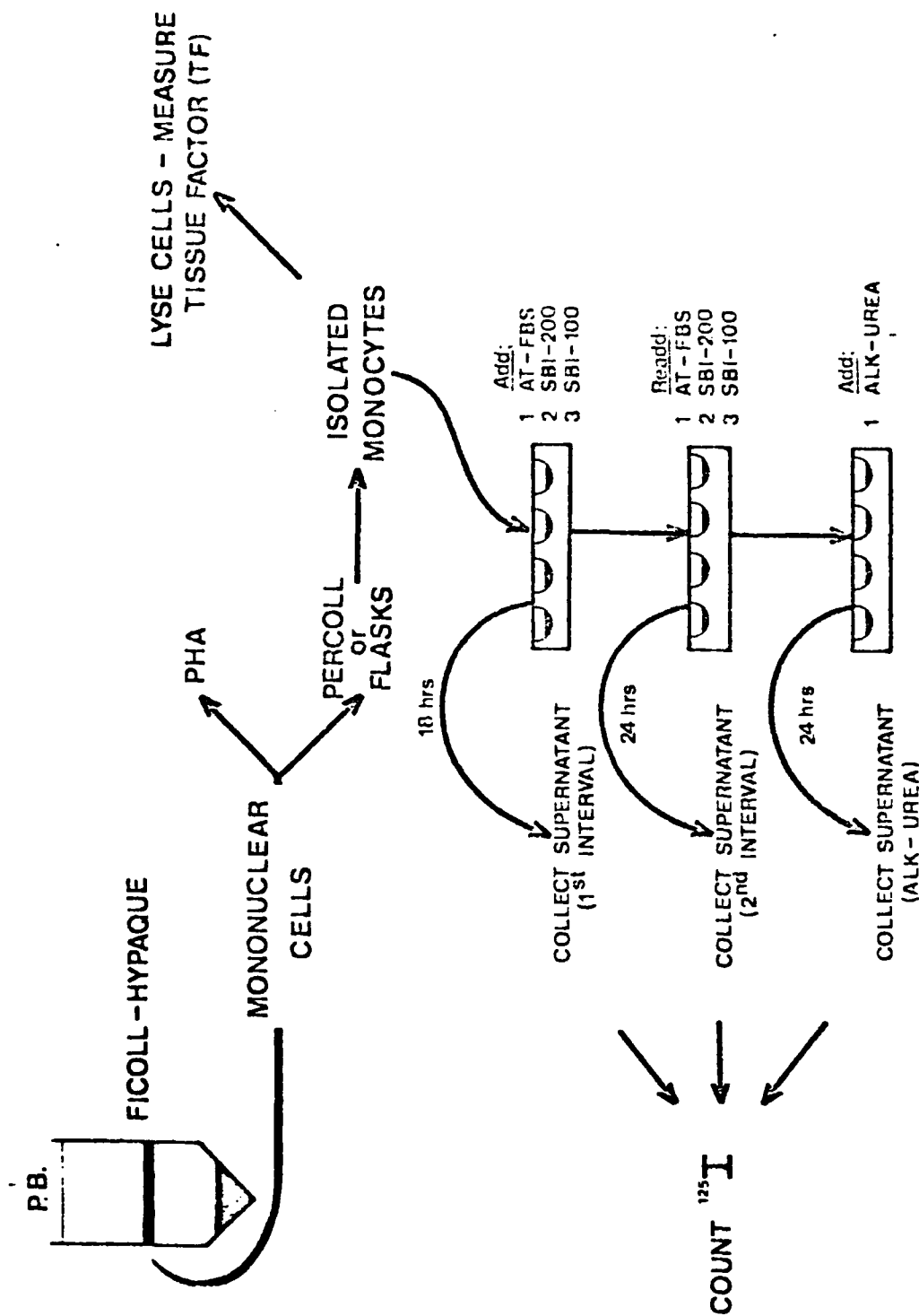
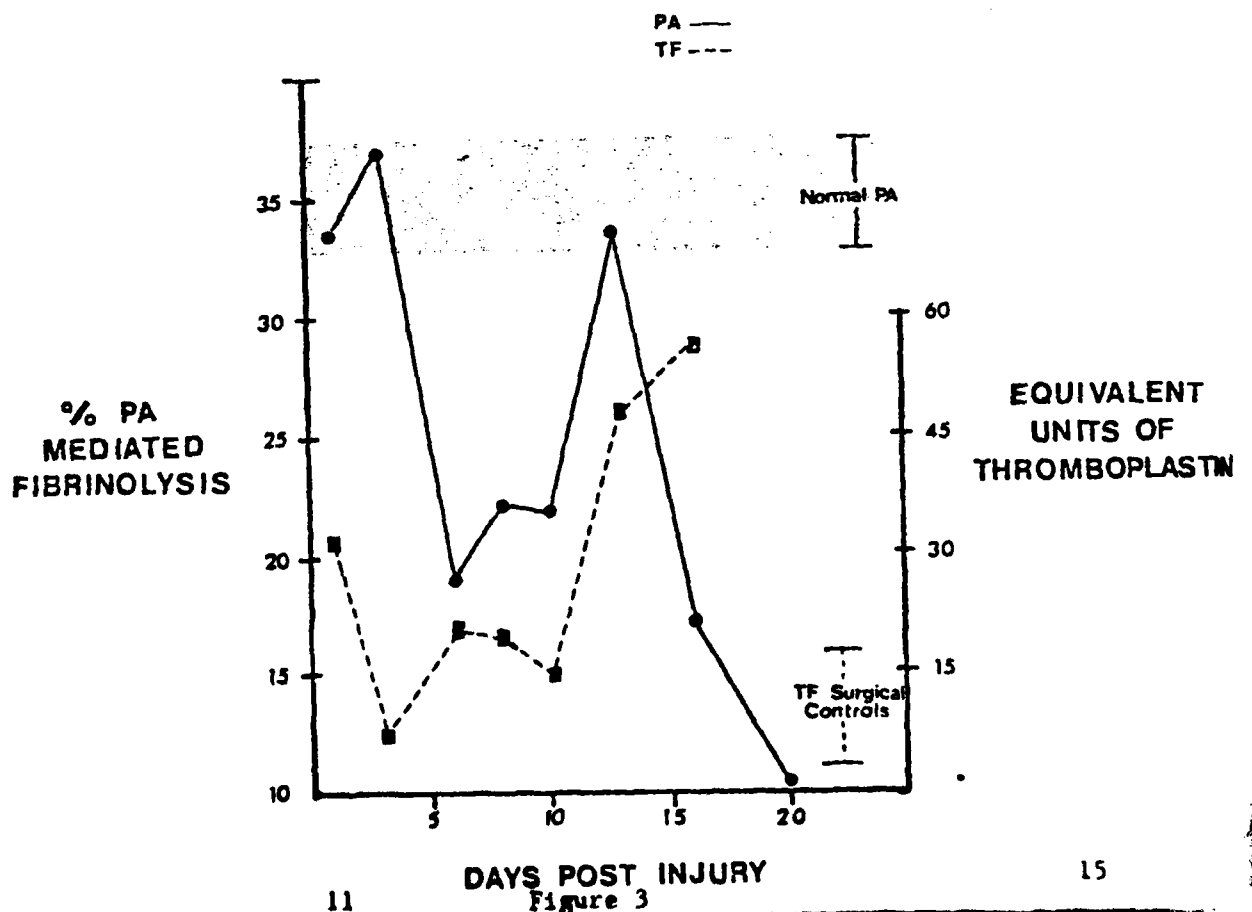
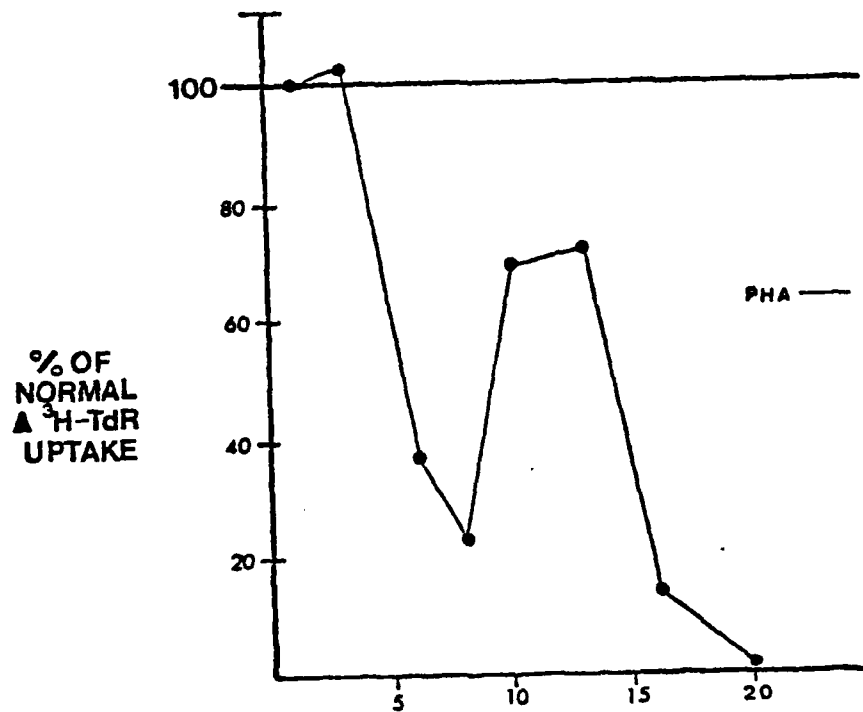


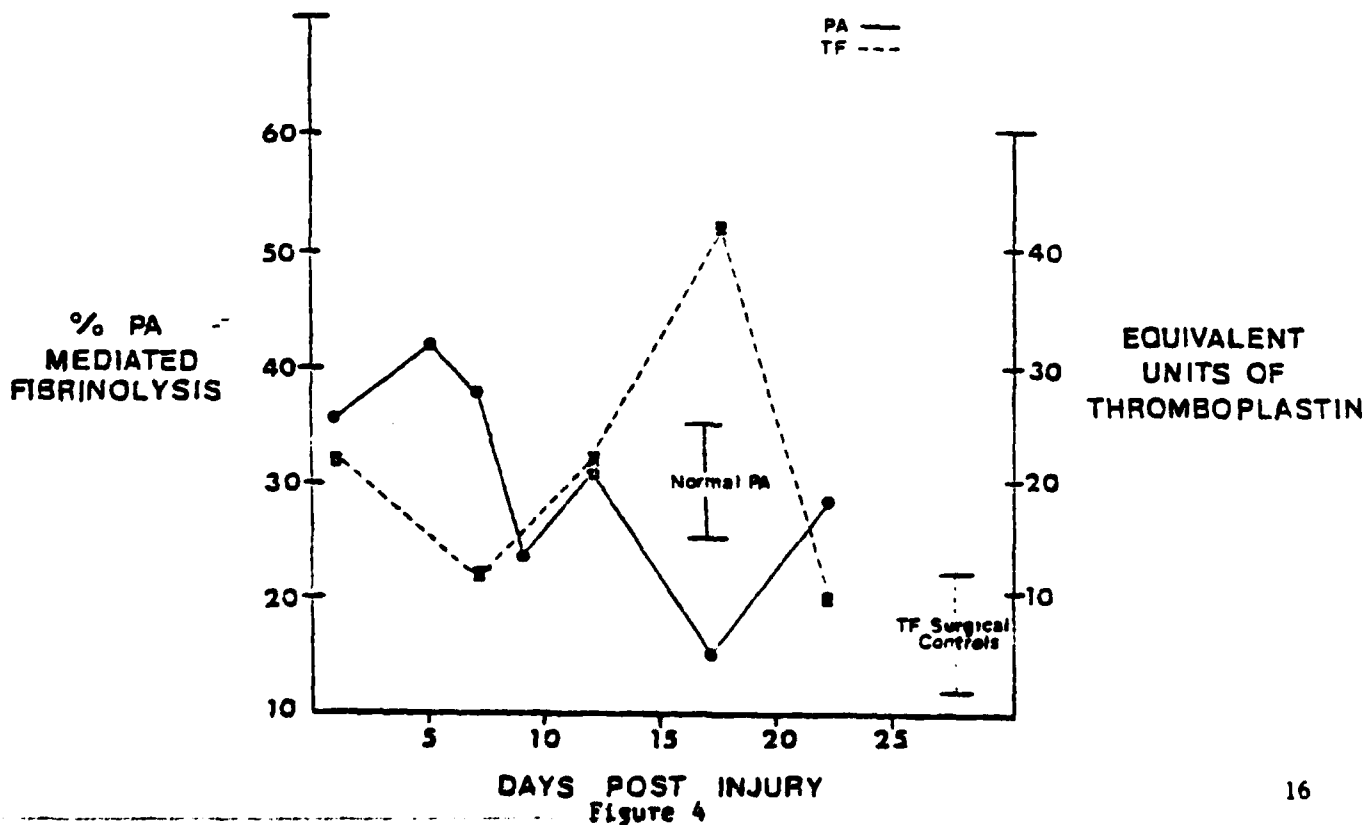
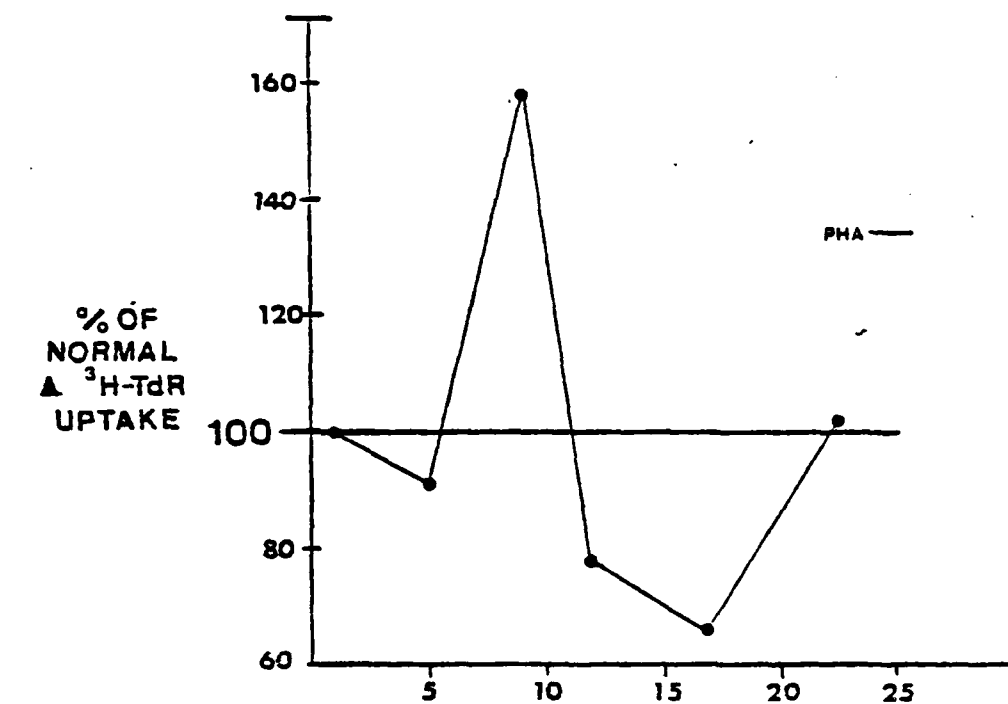
Figure 2

MONOCYTE ACTIVITIES AFTER SEVERE THERMAL TRAUMA



11 DAYS POST INJURY
Figure 3

POST BURN CHANGES IN LYMPHOCYTE AND MONOCYTE ACTIVITIES



CHANGES IN LYMPHOCYTE AND MONOCYTE FUNCTIONS AFTER MAJOR BURNS

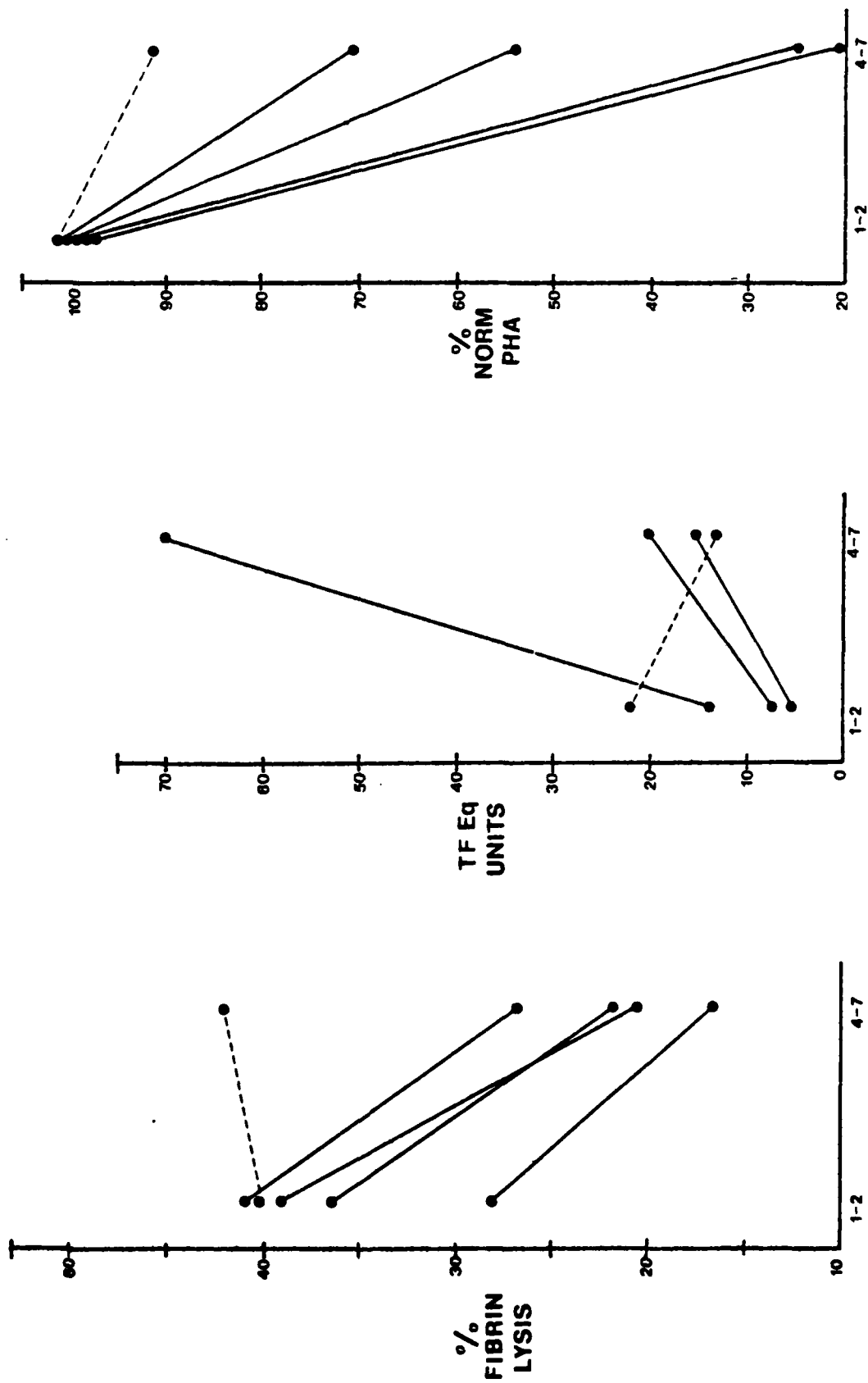


Figure 5

CHANGES IN LYMPHOCYTE AND MONOCYTE FUNCTION AFTER MAJOR SOFT TISSUE DAMAGE

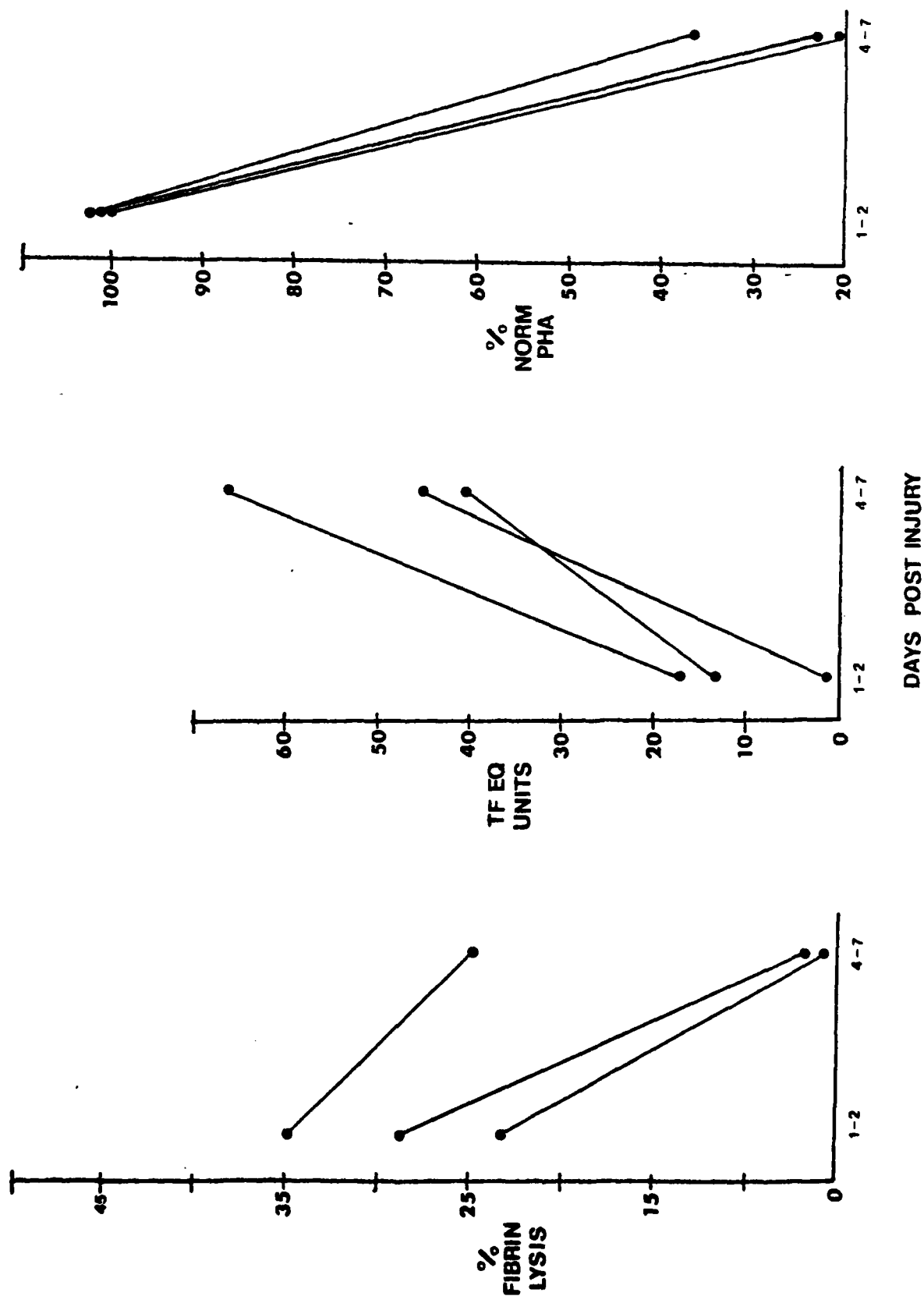


Figure 6

POST INJURY CHANGES IN LYMPHOCYTE AND MONOCYTE ACTIVITIES AFTER SEVERE TRAUMA

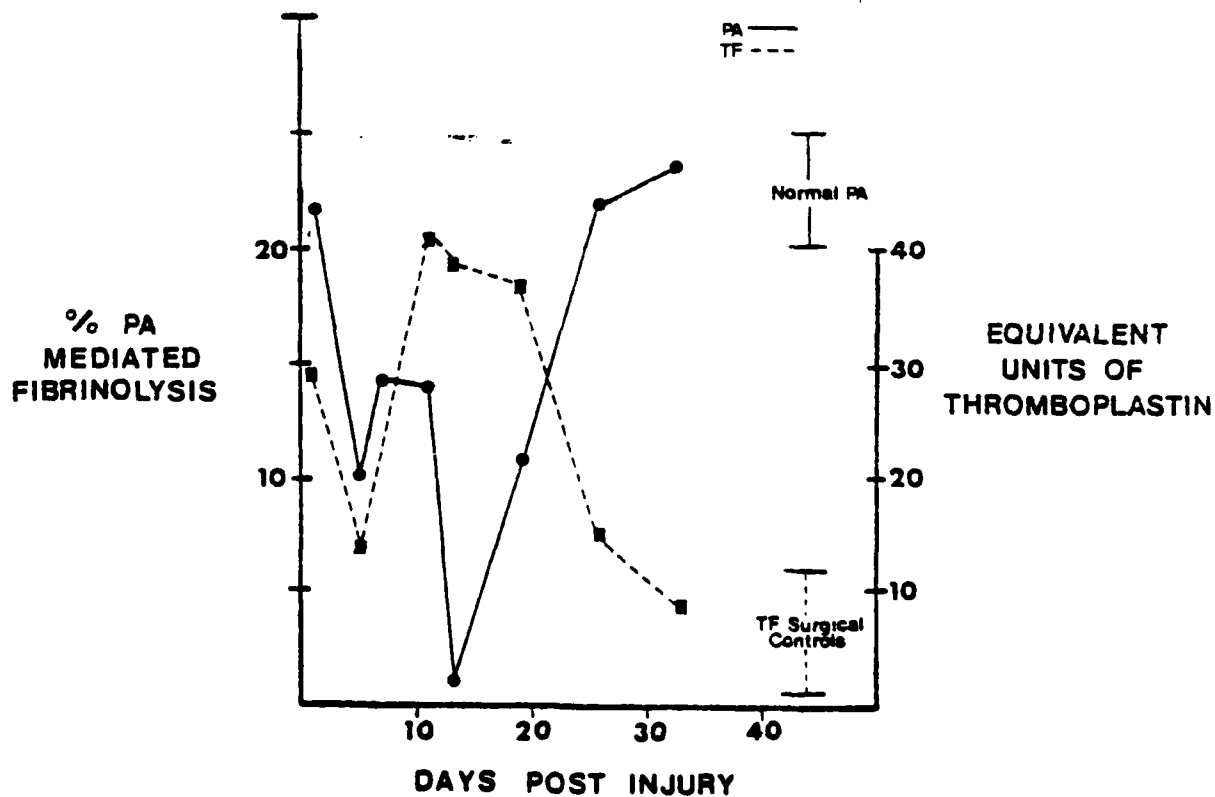
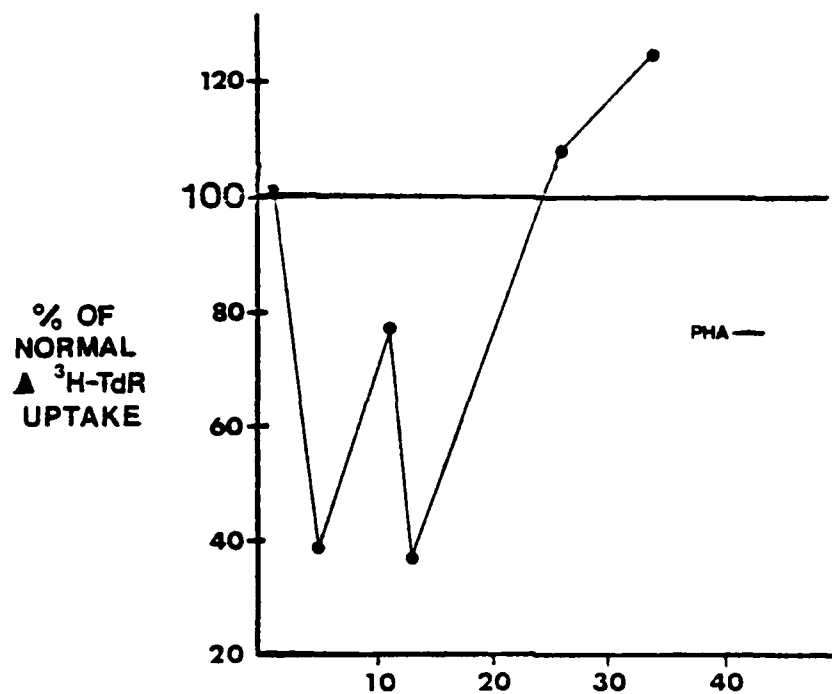


Figure 7

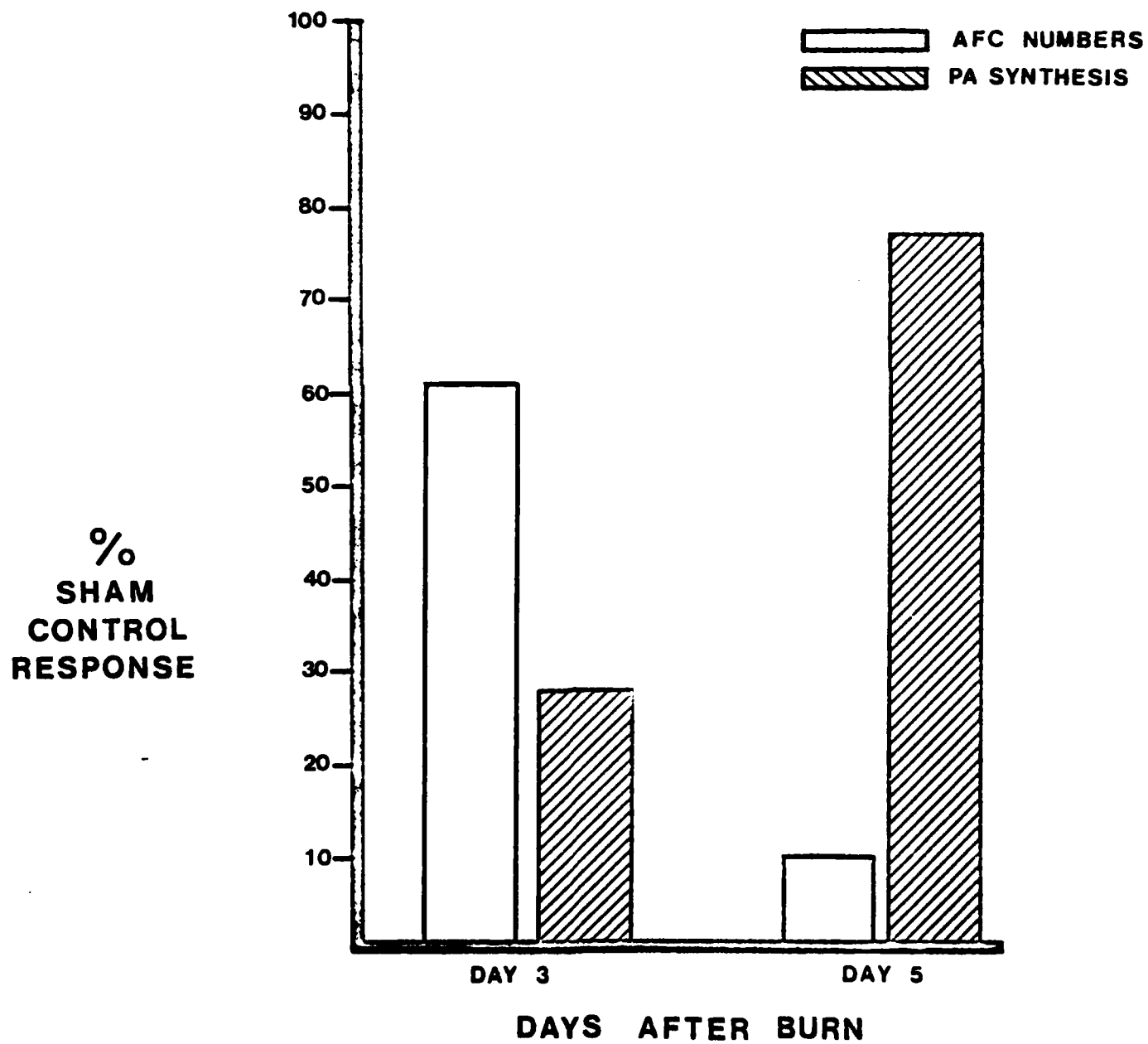


Figure 8

Recent Publications:

Miller, CL: Alterations in macrophage function following thermal injury. In: J. Ninnemann (ed), Immune Consequences of Thermal Injury. Williams and Wilkins, Baltimore, 1980.

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